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Determinants of Human Papillomavirus-Negative, Low-Grade Squamous Intraepithelial Lesions in the Atypical Squamous Cells of Undetermined Significance/Low-Grade Squamous Intraepithelial Lesions Triage Study (ALTS)

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BACKGROUND. Although low-grade squamous intraepithelial lesions (LSIL) most often are the result of infection by human papillomaviruses (HPV), a small proportion of women with LSIL have negative HPV tests. Using the Atypical Squamous Cells of Undetermined Significance/LSIL Triage Study (ALTS) population, the authors evaluated the significance of HPV-negative LSIL.

METHODS. Women with cytologic interpretations of LSIL by referral Papanicolaou (Pap) tests or enrollment ThinPrep tests (range, 1195–1476 women, depending on the specimen type and the reviewer) had HPV testing performed by both Hybrid Capture 2 and polymerase chain reaction (PCR)-based linear array for 27 HPV types.

RESULTS. Using 4 independent cytologic definitions of LSIL, only 3–11% of women with LSIL were found to have HPV-negative results on both HPV tests. The demographic characteristics of women with HPV-negative LSIL were consistent with those of a low-risk population; many were age $>$ 35 years, and many reported no or only 1 recent sexual partner. The absolute risk of a histologic diagnosis of

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cervical intraepithelial neoplasia (CIN) Grade 3/carcinoma during the 2-year trial was lower for women with HPV-negative LSIL (range, 2–4%) compared with the absolute risks for oncogenic HPV-positive women with LSIL (range, 13–19%). However, at the next 6-month follow-up visit, 12%–32% of the women with HPV-negative LSIL had a positive HPV test. Finally, visual inspection of cervigrams demonstrated a clear association between a larger os and negative HPV test results compared with women who had HPV-positive LSIL. This may have influenced HPV sample adequacy.

CONCLUSIONS. Based on the ALTS data, the authors found no evidence to support the existence of HPV-negative LSIL as a distinct biologic entity related to the risk of cervical carcinoma. Such results appear to represent cytologic misinterpretations or falsely negative HPV tests. ***Cancer (Cancer Cytopathol)* 2005;105:253–62.** *Published 2005 by the American Cancer Society*.*

KEYWORDS: human papillomavirus, Hybrid Capture 2, polymerase chain reaction, low-grade squamous intraepithelial lesions, cervical intraepithelial neoplasia.

Infection with 1 of approximately 15 oncogenic human papillomaviruses (HPV) now is recognized as the necessary cause of cervical carcinoma and its precursors.[1–4] This understanding has been translated quickly into clinical practice, with HPV testing now accepted as a means for determining the follow-up of women with atypical squamous cells of undetermined significance (ASCUS) interpretations of their Papanicolaou (Pap) tests.[5] In addition, HPV testing, in conjunction with Pap testing, is an option for the primary screening of women age $\geq$ 30 years.[6]

The Bethesda System for classification of cervical cytology specimens[7–9] consists of a two-tiered terminology for squamous intraepithelial lesions (SIL): low-grade SIL (LSIL) and high-grade SIL (HSIL). The association between SIL and HPV has been documented so well[1] that, currently, it is believed that most (if not all) SIL is HPV-positive. Results from the ASCUS-LSIL Triage Study (ALTS), a clinical trial conducted to determine the optimal follow-up of women who are diagnosed with minimally abnormal Pap tests (ASCUS or LSIL), indicate that the majority of women (82.9%) who were referred with a cytologic diagnosis of LSIL harbored high-risk HPV DNA, as measured by Hybrid Capture 2® (HC2) (Digene Corporation, Gaithersburg, MD) in their enrollment liquid-based cytology specimens.[10] However, it was found that a small proportion of women with a referral cytology interpretation of LSIL had negative HPV results using the HC2 test. Possible explanations for these findings include 1) LSIL caused by nononcogenic HPV types not targeted by HC2, 2) HPV-negative LSIL constituting a true biologic entity, 3) diagnostic misinterpretation of LSIL cytology (false-positive), 4) false-negative HPV tests, or 5) viral clearance between referral and repeat cytology. The objective of the current analysis was to identify the characteristics of women in ALTS with LSIL cytology who were negative for HPV DNA and to determine whether HPV-negative LSIL represents a true biologic entity or a misclassification of cytology and/or HPV test results.

MATERIALS AND METHODS

Study Population

The study design and characteristics of the population within the ALTS trial have been described previously.[10–12] The study was conducted with the approval by local Institutional Review Boards and in accordance with the U.S. Department of Health and Human Services. Briefly, in total, 5060 women who were referred with the interpretation of ASCUS (n = 3488 women) or LSIL (n = 1572 women) on a conventional Pap smear (referral Pap) were enrolled in the study and were assigned randomly to 1 of 3 management strategies: immediate colposcopy, triage based on HPV results and thin-layer cytology results, or triage based on cytology results only. Between November 1996 and December 1998, study enrollment was conducted at four clinical centers: the University of Alabama (Birmingham, AL), Magee-Womens Hospital of the University of Pittsburgh Medical Center Health System (Pittsburgh, PA), the University of Oklahoma (Oklahoma City, OK), and the University of Washington (Seattle, WA). Written informed consent was obtained from each participant. At enrollment, cervical specimens and complete questionnaire data, including demographic, hormone, and sexual histories, were collected. The current analysis was based on all women who were diagnosed with LSIL in referral and/or enrollment cytology specimens. Women were followed every 6 months for 2 years for disease outcomes of histologic cervical intraepithelial neoplasia (CIN) Grade 2 (CIN2) and CIN3/carcinoma.

Definition of LSIL

Women were enrolled into ALTS, on average, 2 months after their original abnormal Pap test ("referral Pap"). At enrollment, a liquid-based cytology specimen (ThinPrep®; Cytyc Corporation, Boxborough, MA) was collected with a broom-type sampler (Papette™ broom; Wallach Surgical Devices, Inc., Orange, CT) and transferred to a PreservCyt® vial (Cytyc Corporation). A second cervical specimen then was collected using a Dacron swab and placed into Specimen Transport Medium™ (STM) (Digene Corporation), which was used for the prototype linear-array polymerase chain reaction (PCR) HPV DNA typing. Finally, acetic acid was applied and a pair of high-resolution photographs of the cervix (Cervigram™; National Testing Laboratories, Fenton, MO) was taken.[13]

The referral Pap smears were read by the community laboratory, and the enrollment ThinPrep Pap slides initially were interpreted by a clinical site pathologist; then, both the referral smears and the enrollment ThinPrep slides were reviewed by the ALTS Pathology Quality-Control (QC) Group.[14] Therefore, for each woman, there were four independently rendered cytologic interpretations: 1) the community laboratory interpretation of the original referral Pap smear, 2) the Pathology QC Group review of the original referral Pap smear, 3) the clinical center interpretation of the enrollment ThinPrep slide, and 4) the Pathology QC review of the enrollment ThinPrep slide. In total, 2546 women were identified with LSIL by any 1 or more of the 4 interpretations, including 2371 women for whom HPV data were available. These 2371 women constituted the population for the current study, and the remaining 175 women were excluded because of missing HPV test results. Because each interpretation was made independently, individual women were included as LSIL by 1 (n = 723), 2 (n = 869), 3 (n = 378), or all 4 (n = 401) of the "definitions" described above. The 401 women who were diagnosed with LSIL by all 4 definitions constituted the consensus LSIL group.

HPV Testing

HPV testing by HC2 was performed from the residual specimen in the enrollment PreservCyt® vial after preparation of the ThinPrep, as described previously.[11,12] Only the high-risk HC2 probe (Probe B) was used to target oncogenic HPV types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68. Women were categorized as either HC2 positive or HC2 negative based on a threshold of 1 pg/mL HPV DNA. In addition to HC2 testing, DNA isolated from each of the enrollment STM specimens was analyzed for 27 individual HPV genotypes using a prototype PCR-based linear-array technique[15,16] (Roche Molecular Systems, Alameda, CA), hereinafter referred to as "PCR." The primers included in this test identified oncogenic HPV types: 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68 (ME180), 73 (MM9, P238A), 82 (MM4, W13B), and 83 (MM7, P291). The nononcogenic HPV types were 6, 11, 26, 40, 42, 53, 54, 55, 57, 66, and 84 (MM8, P155), and β-globin DNA was used as an internal standard.[14] In approximately 40% of the women, an additional 11 HPV types (61, 62, 64, 67, 69, 70, 71, 72, 81, 85, and 91) were tested. Unless otherwise noted, the PCR data presented were restricted to the 27 HPV types that were tested in all women. Multiple HPV types were considered oncogenic if at least one oncogenic HPV type was identified or nononcogenic if only nononcogenic types were found. All cytologic interpretations and HPV tests were performed independently without knowledge of the other results.

Cervigram Evaluation

To address the possibility of visibly evident differences between HPV-negative LSIL and HPV-positive LSIL, we selected a subsample of women for cervigram review.[13] We compared the enrollment cervigrams of 50 randomly selected women whose specimens were positive for HPV (by HC2 and PCR) and had ThinPrep interpretations of LSIL on the enrollment ThinPrep slide (by the clinical center or the Pathology QC Group) with cervigrams from another 50 randomly selected women with LSIL whose specimens concurrently were HPV negative (by HC2 and PCR). We also obtained cervigrams for six of the seven HPV-negative (by HC2 and PCR) women in the consensus LSIL group. One cervigram was not available.

The pair of cervigrams taken from each woman during each patient visit in ALTS, in the form of 35-mm slides, was scanned using a Scanjet ADF scanner (Hewlett Packard, Palo Alto, CA) with a slide adapter at 1660 dots-per-inch resolution and was stored in Tagged Image File Format. The digitized pictures were converted to the Joint Photographic Experts Group format using a compression ratio of 40:1. The digitized pictures were evaluated on a 17-inch color monitor using the Boundary Marking Tool (BMT) software developed by the National Cancer Institute, Division of Cancer Epidemiology and Genetics and the National Library of Medicine.[17] The BMT was used (by J.J.) to select a single image for evaluation. A masked evaluation was performed in which boundaries of the endocervical opening or os, the squamocolumnar junction, and any acetowhite area compat-

ible with CIN were drawn using a Graphire2 mouse (Wacom Technology Corporation, Vancouver, WA). The areas collected were recorded and converted to pixels. In a subsequent, unmasked analysis, the size of the cervix was determined by drawing a boundary around the external borders of the ectocervix.

Disease Outcomes

The women enrolled in the study were monitored every 6 months for 2 years and were censored at the disease outcomes of histologic CIN2 or greater (CIN2, CIN3, or carcinoma). Patients who had outcomes of CIN2 or greater were defined by the clinical center pathology interpretations. A more rigorous endpoint of histologic CIN3/carcinoma as a surrogate for the risk of carcinoma was defined by the Pathology QC Group review.[12]

Statistical Methods

The women who were identified according to the four definitions of LSIL and the women in the consensus LSIL group were stratified by HC2 and PCR HPV test results. For each definition of LSIL, this resulted in six categories of varying HPV status: 1) HC2 positive, oncogenic HPV positive by PCR; 2) HC2 positive, nononcogenic HPV positive by PCR; 3) HC2 positive, HPV negative by PCR; 3) HC2 negative, oncogenic HPV positive by PCR; 5) HC2 negative, nononcogenic HPV positive by PCR; and 6) HC2 negative and HPV negative by PCR. The distribution (number and frequency) of women according to HPV status within each LSIL definition was calculated. In addition, we calculated the absolute risk (positive predictive value) and respective 95% confidence intervals (95% CI) for women within each stratum for developing the histologic outcomes of CIN3/carcinoma (as defined by the Pathology QC Group) and outcomes of CIN2 or greater (as defined by the clinical centers) during the 2-year follow-up.

Subsequent analyses focused on the women identified as HPV negative (i.e., negative by both HC2 and PCR) for each definition of LSIL. For women who were identified with HPV-negative LSIL, the subsequent histories at every 6-month visit (including HC2, PCR, cytology, and pathology results) were examined carefully. Demographic characteristics of the women who were identified with HPV-negative LSIL were investigated, including age, race (non-Hispanic white, Hispanic, black, other), education (less than high school, high school, some college, completed college), age at first intercourse, total number of sexual partners, partners within the past year, smoking behavior (never, former, current), number of Pap tests in the last 5 years, oral contraceptive use within the last 2 years, hormone use, and parity. Moreover, characteristics observed on cervigrams were analyzed as possible predictors of HPV detection. Chi-square statistics were used to evaluate the associations of each characteristic with negative HPV status compared with positive HPV status. Characteristics that were found to be associated in the simple univariate analysis were included in a multivariate model for negative HPV status (by both HC2 and PCR) compared with all other women with LSIL who had positive HPV status, adjusting for study arm and study center and providing adjusted risk estimates (e.g., odds ratio [OR] and 95% CIs). Statistical analyses were performed using the SAS 8.2 (SAS Institute Inc, Cary, NC)and STATA/SE 8.0 (StataCorp LP, College Station, TX) software packages; P values (2-tailed) < 0.05 were considered statistically significant.

RESULTS

In total, 2371 women with available HPV test results were considered LSIL by any 1 or more of the 4 definitions. The number of cytologic interpretations of LSIL rendered by each of the 4 definitions was as follows: 1) community laboratory interpretation of the original referral Pap smear ($n = 1476$ LSIL interpretations), 2) the Pathology QC Group review of the original referral Pap ($n = 1279$ LSIL interpretations), 3) the clinical center interpretation of the enrollment ThinPrep slide ($n = 1244$ LSIL interpretations), and 4) the Pathology QC Group review of the enrollment ThinPrep slide ($n = 1195$ LSIL interpretations) (Table 1). There were 401 women with HPV results who were diagnosed with LSIL according to all 4 definitions, and these women were included in the consensus LSIL group.

The distribution of HPV status among women who had LSIL according to the four definitions and among women in the consensus LSIL group is shown in Table 1. The vast majority of women with LSIL had HPV-positive results according to HC2, PCR, or both tests (89–98%, depending on the definition of LSIL). When considered as separate tests, HC2 and PCR results both were less sensitive, with 84–95% of LSIL identified as positive by HC2 and 83–94% identified as HPV positive by PCR. At least 70% (range, 70–79%) of women had both HC2-positive and oncogenic, HPV-positive PCR results. Less than 15% of women had HC2-positive and nononcogenic, HPV-positive PCR results (range, 8–14%); whereas 4–6% had HC2-positive and PCR-negative results.

A small percentage of women with LSIL had HC2-negative results (range, 5–16%) and could be categorized further in 2 groups: 1) PCR positive and 2) PCR negative. Women with HC2-negative/PCR-positive LSIL accounted for 2–6% of women with LSIL, includ-

TABLE 1
Distribution of Human Papillomavirus Status among Women Diagnosed with Low-Grade Squamous Intraepithelial Lesions, as Defined by Four Distinct Definitions and the Consensus LSIL Group

		LSIL (referral Pap)[a]		LSIL (QC, referral Pap)[b]		LSIL (ThinPrep)[c]		LSIL (QC, ThinPrep)[d]		LSIL (consensus)	
HC2	PCR	No.	%	No.	%	No.	%	No.	%	No.	%
Positive	Onco+	1033	70	901	70	965	78	940	79	311	78
Positive	Nononco+	122	8	120	9	122	10	127	11	55	14
Positive	Negative	86	6	66	5	56	5	58	5	16	4
Negative	Onco+	27	2	29	2	9	1	10	1	4	1
Negative	Nononco+	49	3	45	4	18	1	21	2	8	2
Negative	Negative	159	11	122	10	74	6	40	3	7	2
Total	—	1476	—	1283	—	1244	—	1196	—	401	—

LSIL: low-grade squamous intraepithelial lesion; Pap: Papanicolaou test; QC: quality control; HC2: Hybrid Capture 2 human papillomavirus (HPV) test; PCR: polymerase chain reaction analysis; Onco+: positive for oncogenic human papillomavirus type; Nononco+: positive for nononcogenic human papillomavirus type.

[a] Original referral Papanicolaou test interpretation by the community laboratory.

[b] Pathology quality control review of the referral Papanicolaou test.

[c] Clinical center interpretation of ThinPrep Papanicolaou test.

[d] Pathology quality control review of ThinPrep Papanicolaou test.

[e] Consensus low-grade squamous intraepithelial lesion defined as such by all four reviews.

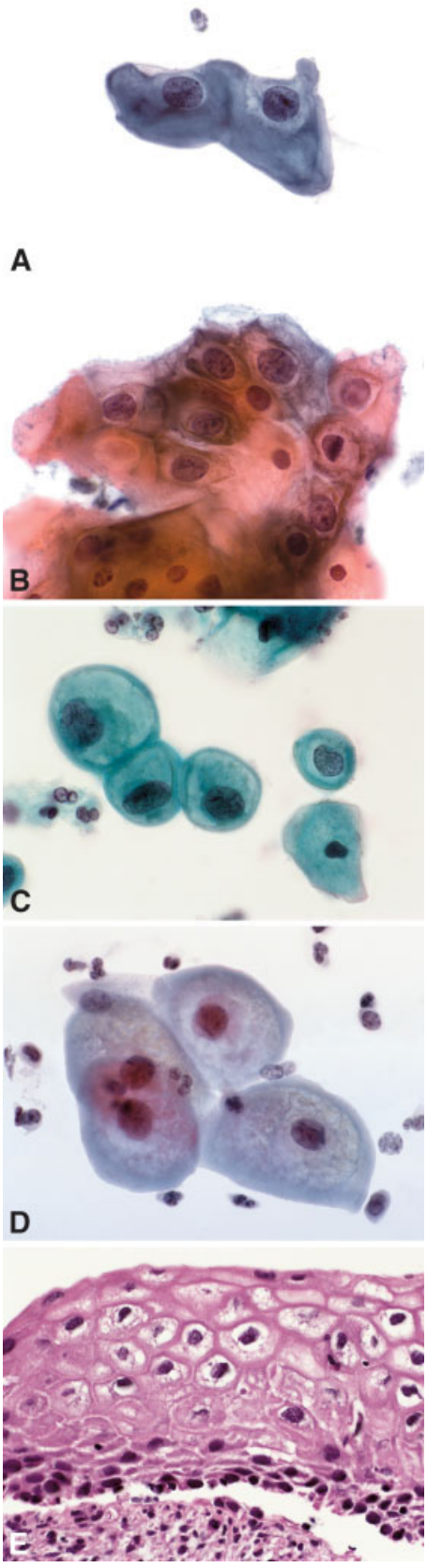
ing women who had positive results for oncogenic HPV types (1–2%) and nononcogenic HPV types (1–4%).

Depending on the definition, 2–11% of LSIL was HPV negative by both HC2 and PCR. This percentage was highest for the community laboratory interpretation of the referral smear, followed, in turn, by the Pathology QC review of the referral smear, the clinical center read, the Pathology QC review of the enrollment ThinPrep slide, and, finally, the consensus LSIL group. It should be noted that HPV tests and the enrollment ThinPrep were performed from samples that were taken concurrently, whereas the referral smear preceded the HPV testing by approximately 2 months. This interval may have allowed time for HPV regression from the time of referral to the enrollment visit.

For women who had HPV-negative results (by HC2 and PCR) with LSIL according to any of the 4 LSIL definitions, we reviewed the visit history during the 2-year follow-up and found that 12–32% of women were positive for oncogenic or nononcogenic HPV types (by HC2 or PCR) at the 6-month visit. Specifically, among the women with HPV-negative LSIL ThinPrep results at enrollment, 12% (by clinical center diagnosis) and 15% (by QC Pathology review) had HPV-positive results at the 6-month visit, whereas the comparable rates for the conventional referral Pap smear were 26% (by clinical center diagnosis) and 32% (by Pathology QC review). Among the women who had HPV-negative results with LSIL, as defined by each of the 4 interpretations, the median age was 5–9 years older for those who remained HPV negative during the 2-year follow-up (ages 28–40 years) compared with women who did not remain HPV negative (ages 22–35 years).

Only 7 women (2%) in the consensus LSIL group (LSIL by all 4 definitions) had HPV-negative results on both HC2 and PCR. Photomicrographs of cytologic changes in the enrollment ThinPrep slides from several of these women in the HPV-negative consensus LSIL group are shown in Figure 1. It is noteworthy that, of the 7 women identified as HPV-negative in consensus LSIL group, review of the visit histories showed that 5 women had HPV-positive test results during the 2-year follow-up, including 3 positive HC2 or PCR results and 2 positive results (1 positive for HPV 64 and 1 positive for HPV 61) by additional PCR testing, as defined by the 11 additional HPV types described above (see Materials and Methods). Of these 5 women with HPV-positive results, 1 woman developed CIN2, and another woman developed CIN3 during follow-up. Thus, there were only two of seven women with no evidence at all of HPV.

Table 2 shows that the absolute risk for developing CIN3/carcinoma during the 2-year ALTS follow-up was highest among women with LSIL who had HPV-positive results by both HC2 and by PCR for oncogenic HPV (range among the 4 LSIL definitions, 13–19%). Although it encompassed a smaller total number of women, the absolute risk for CIN3/carcinoma similarly was high (range, 10–19%) among women who were HC2 negative but oncogenic HPV positive by PCR. Among HC2-positive, PCR-negative women with



LSIL, the risk ranged from 7% to 12%. HC2-negative women with nononcogenic HPV types by PCR were at lower risk (range, 2–6%), but the lowest risk was observed for women who were both HC2 negative and PCR negative (range, 2–4%).

Absolute risks for the less stringent CIN2 or greater outcome are shown in Table 3. Risks were greater when the disease threshold was lowered to CIN2, as expected. However, the correlations remained unchanged.

Investigation of the demographic characteristics of women who were identified with HPV-negative LSIL demonstrated that these women resembled a population at low risk for the development of CIN3/carcinoma compared with HPV-positive women (by HC2, or PCR, or both). For example, when we examined the population of women with LSIL, as defined by the Pathology QC Group review of ThinPrep slides in multivariable analyses, women with HPV-negative LSIL were more likely to be age $\geq$ 35 years (OR, 6.5; 95% CI, 4.2–8.7 compared with women ages 18–19 years), they were more likely to be college graduates (OR, 1.5; 95% CI, 1.1–2.1 compared with women without a high school diploma), and they were more likely to report no recent sexual partners (OR, 2.4; 95% CI, 1.5–3.7 compared with women who had $\geq$ 3 recent partners). Furthermore, among women ages $\geq$ 35

FIGURE 1. These photomicrographs show representative consensus low-grade squamous intraepithelial lesions (LSIL) from patients who had negative human papillomavirus (HPV) test results at their enrollment visit. (A) In a woman age 19 years, rare koilocytes were consistent with LSIL in a sparsely cellular sample. A subsequent Hybrid Capture 2 (HC2) test remained negative, but polymerase chain reaction (PCR) analysis converted to positive at the subsequent 6-month visit. This was interpreted as a false-negative HPV test. (B) In a woman age 21 years, an occasional group of koilocytes was consistent with LSIL. HC2 result was positive at the 12-month visit. Histology at 24 months showed CIN3. This was interpreted as a false-negative HPV test at enrollment. (C) In a woman age 43 years, occasional cells show enlarged, atypical nuclei and cytoplasmic clearing. HC2 result remained negative, but nononcogenic HPV type 61 (not present in the HC2 oncogenic probe mix) was identified at the 6-month visit. (D) In a woman age 39 years, cells with vacuolated cytoplasm and small nuclei were present. HC2 and PCR results consistently were negative throughout follow-up. The patient had no history of oral contraceptive use or other hormone therapy. Note the intracytoplasmic glycogen with "cracking artifact," as described by Morrison et al.[19] This was interpreted as a false-positive cytology result with "pseudokoilocytosis" due to intracytoplasmic glycogen (Papanicolaou stain). (E) A concurrent biopsy from the same woman shown in Panel D was diagnosed as CIN1 by both the clinical center and QC pathologists. Note the lace-like, fragmented cytoplasm, which may be the tissue counterpart to the glycogen "cracking artifact" noted in Panel D (H & E). This was interpreted as a false positive histologic diagnosis of CIN1. Original magnification $\times$ 63 (A–D); $\times$ 40 (E).

TABLE 2
Absolute Risk for a Pathology Quality Control Review Diagnosis of Cervical Intraepithelial Neoplasia Grade 3/Carcinoma According to Human Papillomavirus Status in Women Diagnosed with Low-Grade Squamous Intraepithelial Lesions, as Defined by Four Distinct Definitions and the Consensus LSIL Group

		LSIL (referral Pap)[a]		LSIL (QC, referral Pap)[b]		LSIL (ThinPrep)[c]		LSIL (QC, ThinPrep)[d]		LSIL (consensus)[e]	
HC2	PCR	CIN3+ (no./total)	Risk (95% CI)	CIN3+ (no./total)	Risk (95% CI)	CIN3+ (no./total)	Risk (95% CI)	CIN3+ (no./total)	Risk (95% CI)	CIN3+ (no./total)	Risk (95% CI)
Positive	Onco+	201/1033	19 (17–22)	143/901	16 (14–18)	137/965	14 (12–17)	124/940	13 (11–16)	29/311	9 (6–13)
Positive	Nononco+	5/122	4 (1–9)	5/120	4 (1–9)	3/122	2 (1–7)	4/127	3 (1–8)	3/55	5 (1–15)
Positive	Negative	8/86	9 (4–18)	8/66	12 (5–22)	4/56	7 (2–17)	4/58	7 (2–17)	1/16	6 (0–30)
Negative	Onco+	5/27	19 (6–38)	3/29	10 (2–27)	1/9	11 (0–48)	1/10	10 (0–45)	1/4	25 (1–81)
Negative	Nononco+	1/49	2 (0–11)	1/45	2 (0–12)	1/18	6 (0–27)	1/21	5 (0–24)	1/8	13 (0–53)
Negative	Negative	5/159	3 (1–7)	3/122	2 (1–7)	3/74	4 (1–11)	1/40	3 (0–13)	1/7	14 (0–58)

LSIL: low-grade squamous intraepithelial lesion; Pap: Papanicolaou test; QC: quality control; HC2: Hybrid Capture 2 human papillomavirus (HPV) test; PCR: polymerase chain reaction analysis; CIN3 +: Grade 3 or carcinoma; 95%; CI: 95%; confidence interval; Onco+: positive for oncogenic human papillomavirus type; Nononco+: positive for nononcogenic human papillomavirus type.

[a] Original referral Papanicolaou test interpretation by the community laboratory.

[b] Pathology quality control review of the referral Papanicolaou test.

[c] Clinical center interpretation of ThinPrep Papanicolaou test.

[d] Pathology quality control review of ThinPrep Papanicolaou test.

[e] Consensus low-grade squamous intraepithelial lesion defined as such by all four reviews.

TABLE 3
Absolute Risk for a Diagnoses of Cervical Intraepithelial Neoplasia Grade 2 or Greater by the Clinical Centers According to Human Papillomavirus Status in Women Diagnosed with Low-Grade Squamous Intraepithelial Lesions, as Defined by Four Distinct Definitions and the Consensus LSIL Group

		LSIL (referral Pap)		LSIL (QC, referral Pap)[b]		LSIL (ThinPrep)[c]		LSIL (QC, ThinPrep)[d]		LSIL (consensus)[e]	
HC2	PCR	CIN2+ (no./total)	Risk (95% CI)	CIN2+ (no./total)	Risk (95% CI)	CIN2+ (no./total)	Risk (95% CI)	CIN2+ (no./total)	Risk (95% CI)	CIN2+ (no./total)	Risk (95% CI)
Positive	Onco+	330/1033	32 (29–35)	261/901	29 (26–32)	258/965	27 (24–30)	254/940	27 (24–30)	66/311	21 (17–26)
Positive	Nononco+	19/122	16 (10–23)	20/120	17 (10–25)	15/122	12 (7–19)	19/127	15 (9–22)	9/55	16 (8–29)
Positive	Negative	12/86	14 (7–23)	11/66	17 (9–28)	8/56	14 (6–26)	9/58	16 (7–27)	2/16	13 (2–38)
Negative	Onco+	5/27	19 (6–38)	3/29	10 (2–27)	1/9	11 (0–48)	1/10	10 (0–45)	1/4	25 (1–81)
Negative	Nononco+	2/49	4 (0–14)	4/45	9 (2–21)	1/18	6 (0–27)	2/21	10 (1–30)	1/8	13 (0–53)
Negative	Negative	13/159	8 (4–14)	8/122	7 (3–13)	4/74	5 (1–13)	2/40	5 (1–17)	2/7	29 (4–71)

LSIL: low-grade squamous intraepithelial lesion; Pap: Papanicolaou test; QC: Quality Control; HC2: Hybrid Capture 2 human papillomavirus (HPV) test; PCR: polymerase chain reaction analysis; CIN2+: Grade 2 or greater cervical intraepithelial neoplasia; 95% CI: 95% confidence interval; Onco+: positive for oncogenic human papillomavirus type; Nononco+: positive for nononcogenic human papillomavirus type.

[a] Original referral Papanicolaou test interpretation by the community laboratory.

[b] Pathology quality control review of the referral Papanicolaou test.

[c] Clinical center interpretation of the enrollment ThinPrep Papanicolaou test.

[d] Pathology quality control review of the enrollment ThinPrep Papanicolaou test.

[e] Consensus low-grade squamous intraepithelial lesion defined as such by all four reviews.

years with LSIL, oral contraceptive and/or hormone use did not appear to be associated with HPV status, although other authors have reported that hormone therapy is associated with cytologic artifacts that resemble HPV effects.[18,19]

Of the 107 cervigrams that were selected for the current evaluation, 103 cervigrams were adequate, 3 cervigrams were inadequate for evaluation, and 1 cervigram was not available. There were 54 women with negative HPV tests (mean ± standard deviation [SD] age, 38.8 ± 11.7 years; range, 19–66 years), and the mean ± SD parity was 2.1 ± 1.59 children (range, 0–9 children). The HPV-positive group included 49 women (mean ± SD age, 24.2 ± 5.35 years; range, 18–46 years), and the mean ± SD parity was 0.76 ± 0.95 children (range, 0–3 children). Parity was related to age, as expected. Therefore, to control for the confounding influences of age and parity, subsequent analyses were adjusted for age.

We found acetowhite areas compatible with CIN

TABLE 4
Cervigram Review of 50 HC2-Negative/PCR-Negative and 50 HC2-Positive/PCR-Positive Women Diagnosed with LSIL on Enrollment ThinPrep Tests by Clinical Center or Pathology QC Group Interpretation

Measured area	HPV+	HPV−	OR[a]	95% CI
Os grouping				
1 (smallest)	23	12	1.00	
2	18	15	1.07	0.27–4.31
3 (largest)	8	27	0.17	0.03–0.93
SCJ (area of ectopy)				
0 (not visible)	16	27	1.00	
1 (small ectopy)	18	12	0.86	0.22–3.40
2 (big ectopy)	15	15	1.13	0.23–5.64

HPV: human papillomavirus; +: positive; −: negative; OR: odds ratios; 95% CI: 95% confidence interval; SCJ: squamocolumnar junction; LSIL: low-grade squamous intraepithelial lesions. HC: hybrid capture; PCR: polymerase chain reaction; QC: quality control.
[a] Odds ratio (adjusted for age) for human papillomavirus-positive status among women with low-grade intraepithelial lesions according to anatomic characteristics.

in 18 of 54 women (33%) with HPV-negative LSIL and in 25 of 49 women (51%) with oncogenic HPV-positive LSIL ($P = 0.07$). Table 4 shows the associations of measurement of the os and of the ectopy (glandular epithelium visible in ectocervix) areas with HPV positivity. We found a clear association between a large os and negative HPV tests (OR, 0.17; 95% CI, 0.03–0.93). The area of ectopy was not found to be related to HPV positivity. Subsequently, we performed an unmasked evaluation of the size of the entire ectocervix (data not shown) and found that a larger ectocervix was related to HPV-negative results (P trend = 0.002). A cervigram from a woman with LSIL who had HC2-negative and PCR-negative results at enrollment are shown in Figure 2; extensive acetowhitening is observed in the upper lip. However, the os of the cervix, where the HPV sample likely was taken, is far from the acetowhite epithelium, suggesting the possible suboptimal collection of cells from the area of CIN.

DISCUSSION

The current results reaffirm the strong association of LSIL with HPV positivity in general and specifically for the 13 oncogenic HPV types as defined here. Of note, approximately 10% of LSIL was HC2 positive but PCR positive only for non-oncogenic HPV types, a reflection of the known cross-reactivity of HC2 probe B for some low risk HPV types.[10] Using four different definitions of LSIL based on two separate cytology specimens (the referral smear and the enrollment liquid-based ThinPrep) and interpretations rendered by different groups of pathologists (community laboratory, clinical center, and Pathology QC Group), a small

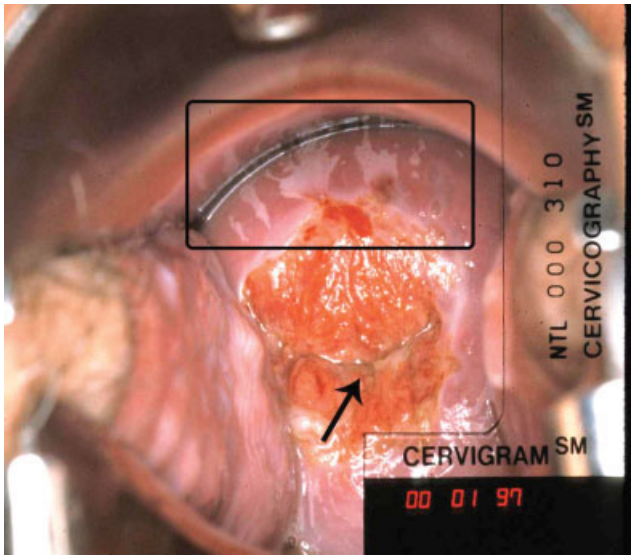


FIGURE 2. In this cervigram from a patient with a human papillomavirus-negative, low-grade squamous intraepithelial lesion (as determined both by Hybrid Capture 2 and polymerase chain reaction analysis) at enrollment, an extensive acetowhite area is observed on the upper lip (black box). The cervical os (arrow), from which the sample was taken, is far from the dysplastic epithelium.

percentage of LSIL was negative for HPV by both HC2 and PCR. One-third of these women had an impression of CIN on their enrollment cervigrams, and some of them had positive HPV tests or CIN2–CIN3 in follow-up visits, suggesting false-negative enrollment HPV tests. Thus, the number of remaining women with HPV-negative LSIL was extremely small. Possible explanations for these results include interpretive error in the cytologic assessment or the involvement of rare, low-risk HPV types that were not detected by either of the HPV tests.

The percent of LSIL that was HPV negative (by both HC2 and PCR) varied by cytology definition; the community laboratory interpretation of the original Pap smear had the highest percent of HPV-negative LSIL (11%); followed, in turn, by the Pathology QC Group review of the original smear; the clinical center interpretation of the enrollment ThinPrep slide; the Pathology QC Group review of the ThinPrep slide; and, finally, the consensus LSIL diagnosis, with only 2% HPV negative. These findings likely are associated with the timing of the sample collection and with differences in the interpretative thresholds used by different groups of pathologists. First, the referral Pap smear preceded the enrollment HPV test by 2 months on average, allowing time for regression of some lesions and therefore accounting for the higher percent of HPV-negative LSIL on referral smears compared with

enrollment ThinPrep slides, which were concurrent with enrollment HPV samples. Second, the Pathology QC Group may have applied more stringent criteria for LSIL in the context of ALTS research compared with the community and clinical center pathologists, who were responsible for patient care. The lowest percent of HPV-negative LSIL (2%) was observed for women diagnosed with cytologic LSIL by all 4 definitions, demonstrating the increased specificity achieved with consensus among multiple pathologist groups. Our analysis of demographic factors, follow-up HPV tests, and enrollment cervigrams of women suggest two different factors that comprise the small fraction of HPV-negative LSIL: false-positive cytologic interpretations of LSIL and false-negative HPV test results.

In terms of diagnostic reproducibility, LSIL is one of the most robust of cytologic interpretations.[18] However, mimics of HPV-associated koilocytosis reportedly include intracytoplasmic glycogen, particularly in women taking oral contraceptives[19] or hormone-replacement therapy,[20] or the so-called "pseudokoilocytosis" identified in some older women with atrophic patterns.[21] We found that women in the ALTS population who had HPV-negative LSIL demographically resembled a lower risk population—more likely to be older, to be college graduates, and to report fewer sex partners—compared with women who had HPV-positive LSIL, supporting the view that some percent of HPV-negative LSIL represents false-positive cytology. However, we found no association with oral contraceptives or hormone use in this population.

Our analyses of absolute risk among women with HPV-negative LSIL demonstrated low absolute risks for both CIN3/carcinoma and CIN2 or greater outcomes. However, it is interesting to note that this risk was not zero, which would be expected for a cohort of women who truly are negative for HPV. In addition, from 12–32% of with women HPV-negative LSIL had a positive HPV test at the next 6-month follow-up visit. Both the nonzero risk for CIN3/carcinoma and the rate of "conversion" to positive HPV status support the view that some HPV-negative LSIL reflects false-negative enrollment HPV test results. False-negative HPV test results may be due to vagaries of sample collection, technical problems with the assay process, or the presence of rare, low-risk HPV types that are not detected by the current tests. We conclude that even a highly sensitive test like that for HPV DNA will not achieve perfect sensitivity.

We found that the size of the os and the size of the entire ectocervical area were significantly larger in women who had negative HPV results. We conclude that some of those women had false-negative enrollment HPV test results, possibly due to sampling error. However, cytologic abnormalities were identified in these women, so that diminished cellular exfoliation[22] does not appear to be the straightforward explanation for the negative HPV tests.

It is important to note that approximately 25% of women with cytologic LSIL actually harbor a high-grade lesion $\geq$ CIN2.[14] Cytologic under diagnosis of high-grade lesions is associated with small numbers of abnormal cells in the cytology sample.[23] American Society for Colposcopy and Cervical Pathology management guidelines recommend colposcopy for LSIL primarily to exclude a higher grade lesion.[5,6] HPV triage is not recommended for LSIL because of the high numbers of women who would be identified as HPV positive. However, with the option to use HPV testing as an adjunct to the Pap test in screening women age $\geq$ 30 years, it is inevitable that HPV-negative LSIL will arise in the routine clinical setting. Although The ALTS population had only modest numbers of women age $\geq$ 30 years with HPV-negative LSIL, the risk for $\geq$ CIN2 was low, ranging from 0% to 4%, depending on the cytologic definition of LSIL (data not shown). Until additional data are available, we believe that women age $\geq$ 30 years with HPV-negative LSIL should continue to be followed according to the current guidelines.[6]

Overall, the current results in the ALTS population confirm that approximately 75–80% of women with cytologic diagnoses of LSIL harbor oncogenic HPV types. Another 10–15% of LSIL is associated with non-oncogenic HPV types. Only about 5–10% of LSIL is negative for HPV. The current findings do not support the existence of HPV-negative LSIL as a true biologic entity. Rather, it appears that this group represents either an interpretive error in the cytologic assessment or a false-negative HPV test.

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